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Original Research Article

Characterization, purity assessment, and preparation of liposomal formulation of 2,4,6-trihydroxygeranylaceto-phenone

Yamen Alkhateeb[1], Qais B Jarrar[2], Faridah Abas[3], Yaya Rukayadi[3], Khozirah Shaari[1,4]*

[1]Laboratory of Natural Product, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, [2]Department of Applied Pharmaceutical Sciences, Faculty of Pharmacy, Al-Isra University, Amman, Jordan, [3]Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, [4]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

For correspondence:* ***Email:* *khozirah@upm.edu.my;* ***Tel:*** *(+603) 97691481*

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Abstract

Purpose: *To prepare, characterize, and determine the purity of 2,4,6-trihydroxygeranylacetophenone (tHGA), and also to develop and characterize a liposomal formulation of tHGA to overcome its poor water solubility.*

Methods: *The tHGA was synthesized and then purified in two steps using two types of column chromatography separation techniques. The compound was characterized using different analytical techniques, while the purity of tHGA was determined by quantitative-nuclear magnetic resonance (qNMR). Proliposomes method was developed to produce liposome-encapsulated tHGA which was characterized based on particle size, polydispersity, stability, and encapsulation efficiency. A selective and rapid high-performance liquid chromatography (HPLC) method was developed and validated to quantify tHGA in a liposomal formulation in order to evaluate the encapsulation efficiency.*

Results: *The tHGA was successfully prepared and characterized with 98.4 % purity. A simple and reproducible proliposomes method was successfully developed to produce liposome-encapsulated tHGA. The liposomal formulation exhibited excellent encapsulation efficiency (90.4 %). This formulation also yielded a homogenous liposome population (polydispersity index = 0.39) with a small particle size (250.8 nm). The prepared liposome-encapsulated tHGA was stable at refrigerated temperature (4 ºC) for at least four weeks. The developed HPLC method showed good linearity over the range of 10 to 500 µg/mL with high precision and accuracy.*

Conclusion: *The compound produced has a high purity which can be used as an analytical reference standard. The developed formulation is effective for dissolving and entrapping a high amount of tHGA which helps to overcome its poor solubility.*

Keywords: *Liposomes, Proliposomes, Trihydroxygeranylacetophenone, tHGA, Spectroscopic characterization, Poor solubility*

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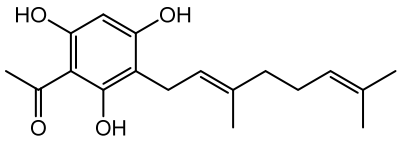
INTRODUCTION

Natural products have unique and massive chemical diversity, making them major sources of medicinal products for centuries. However, the natural compounds could exist in small amount in a plant which limits its availability. Additionally, the isolation and purification of the natural compound from its complex mixture in the natural source is inclined to be difficult, slow, and costly [1]. Some natural products can be prepared easily and cost-effectively via chemical synthesis to overcome the limitations. The synthesis of natural products has had the greatest role behind the natural products contributions in drug discovery [2]. In order to use the synthesized compound as a reference standard for analytical methods, it has to be in the form of the highest purity compound which can be achieved using reasonable effort, and carefully characterized [3].

2,4,6-trihydroxy-3-geranyl acetophenone (tHGA) (Figure 1) is a bioactive compound originated from *Melicope ptelefolia* leaves [4]. The pharmacological investigations on tHGA conducted by our research team have established its anti-inflammatory activities *in vitro* and *in vivo* [5-13]. The dual inhibition of cyclooxygenase-2 and 5-lipoxygenase makes tHGA an interesting anti-inflammatory and anti-allergic agent with fewer side effects which is usually associated with the classic anti-inflammatory drugs [14]. However, the poor water solubility of tHGA complicates the drug delivery process and may affect its oral bioavailability, which can limit its pharmacological function after oral administration.

One of the most popular formulations in drug delivery systems in recent years is liposomal formulation due to its similarity to biological membranes as well as its ability to entrap various substances with different polarities. Liposomes are spherical vesicles with an aqueous core enclosed by one or more phospholipid bilayers [15]. Proliposomes method is widely used for the preparation of liposomes drug delivery system because it is rapid, cost-effective, and does not require excessive manipulation. According to many published reports, proliposomes are suitable to encapsulate both hydrophilic and hydrophobic drugs with high encapsulation efficiencies [16-18].

In this study, tHGA was synthesized and then purified in two steps using two types of column chromatography separation techniques namely open column chromatography over normal phase and reversed phase semi-preparative HPLC. The compound was characterized using proton and carbon nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), infrared spectroscopy (IR), and ultraviolet-visible spectroscopy (UV-Vis) to confirm its identity and quality. The purity of tHGA was determined by quantitative-NMR (qNMR). In addition, a liposomal drug delivery system for tHGA was prepared using the proliposomes method and different analytical techniques were applied and validated to characterize the liposomal formulation of tHGA based on morphology, size of particles, polydispersity, stability, and encapsulation efficiency.



tHGA

Figure 1: Chemical structure of 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA)

EXPERIMENTAL

Chemicals and reagents

Phloracetophenone (purity ≥ 99.0%), anhydrous potassium carbonate, silica gel 60, TLC plates, and deuterated methanol were purchased from Merck, Germany. Geranyl bromide (95% purity) and $DMSO_2$ (99.96% Purity) were purchased from Sigma Aldrich, USA. (ProlipoTM Duo) was purchased from Lucas Meyer, France while Dimethylsulfoxide (DMSO) was purchased from Sigma Aldrich, USA. Ultrapure water for HPLC was taken from Sartorius Arium 611DI ultra-pure water purification system (Sartorius Stedim Biotech, Goettingen, Germany) and Acetonitrile (HPLC grade) was purchased from Fisher Scientific, USA.

Preparation of tHGA

The compound was prepared according to a described method (Ismail *et al* 2012) via the reactions of phloracetophenone (1.000 g, 6 mmol), geranyl bromide (0.876 g, 4.80 mmol), and anhydrous potassium carbonate (0.415 g, 3.00 mmol) in dry acetone (3.5 mL). The mixture was stirred well and then refluxed for 6 hours. The reaction mixture was filtered and evaporated under reduced pressure.

Purification of tHGA

Open column chromatography was performed on a glass column over silica gel 60 (300g 70-230

mesh). The column was prepared by pouring silica gel into the column, with a ratio of approximately 1:10 to pack silica sample. The top of the packed silica was covered by a layer of sand before the sample was introduced into the column. The sample was dry-packed with silica gel and placed on the top of the sand layer. The column was eluted using n-hexane and then n-hexane containing increasing amounts of ethyl acetate, to elute tHGA. Thin layer chromatography (TLC) was used to assess the purity of the eluted compound under UV light to visualize the compound.

The compound was further separated using reversed phase HPLC to obtain high purity tHGA. The chromatographic conditions were as follows: System: JASCO LC-2000 Plus series (Jasco, Japan) equipped with binary pump (PU 2086), solvents mixer (MX-2080-32), photodiode array detector ranging from 195 to 650 nm (MD-2010), autosampler with variable injection volume from 0.1 to 200 µL (AS-2055), and a chromatography data collector (LC-Net-II/ ADC), column: Zorbax SB-C18 (9.4 x 150 mm, 5 µm), flow rate: 4 mL/min, injection volume:100 µl and gradient elution for mobile phase consisted of acetonitrile and water.

Characterization

The structure of tHGA was characterized to confirm its identity by applying several techniques (melting point, NMR, FT-IR, UV/Vis, and MS/MS).

NMR assessment was accomplished by using Varian INOVA 500 NMR spectrometer (Varian Inc., Palo Alto, California, USA), running at a base frequency of 500 MHz for ^{1}H-NMR at 24°C with a number of scans = 64, relaxation delay = 1 s, and pulse angle = 45°. ^{13}C-NMR had base frequency of 125 MHz at 24 °C with a number of scans = 5000, relaxation delay = 1 s, and pulse angle = 45°. Chemical shifts were reported in parts per million (ppm) on the δ scale and were referenced to the TMS signal. Coupling constants (J) was reported in Hz.

FTIR spectrum was collected using Fourier transform spectrometer, Nicolet 6700, equipped with attenuated total reflectance (ATR) attachment (Thermo Scientific, USA).

The UV/Vis spectrum of tHGA has been recorded for the determination of λ_{max} in the region of 200–600 nm using Dionex Ultimate 3000 apparatus (Dionex Corporation, USA) equipped with a PDA 3000 photodiode array detector.

Mass spectrometry was conducted on a high-resolution Q Exactive™ quadrupole-orbitrap mass spectrometer equipped with a heated electrospray ion (H-ESI) source in negative-ion mode (Thermo Scientific) and attached to an LC system Ultimate™ 3000 (Dionex; Sunnyvale, California, USA). Data were acquired with Xcalibur software 4.0 (Thermo Scientific).

Purity assessment

The purity of tHGA was assessed using qNMR with dimethylsulfone ($DMSO_2$) as an internal calibrant (IC). The purity of tHGA was determined from the relation between the areas under the spectral peaks generated from tHGA and $DMSO_2$, the proton number, the prepared masses, and the molecular weights of tHGA and $DMSO_2$ using Equation 1 [19].

$$P[\%] = \frac{n_{IC}, Int_t, MW_t, m_{IC}}{n_t, Int_{IC}, MW_{IC}, M_s}, P_{IC} \quad \ldots\ldots\ldots (1)$$

where m_{IC} and m_S are the amount taken for the internal calibrant (IC) and sample, respectively. Int_{IC} and Int_t are the peak area (integral) of the IC and analyte (t) resonance signals used for quantification, respectively. n_{IC} and n_t are the number of protons that give rise to Int_{IC} and Int_t, respectively. MW_{IC} and MW_t are the molar mass of the internal calibrant and tHGA, respectively, while P_{IC} is the purity of the internal calibrant as a percent value.

HPLC method validation

JASCO LC-2000 Plus series (Jasco, Japan) was the HPLC instrument used in this study. The chromatographic separation was performed using a C18 column, Supelco (150 x 2.1mm, 5µm) (Sigma-Aldrich, USA). A good peak shape of tHGA (Rt 4.1 minutes) was achieved with a mobile phase consisting of acetonitrile-water (60:40) over a total run time of 5.5 minutes. The wavelength was set to 290 nm, with the flow rate and injection volume of 1 mL/minute and 20 µl, respectively.

A stock solution of tHGA (0.5 mg/mL) was prepared by dissolving tHGA in the mobile phase. Serial dilutions with the mixture were conducted to obtain five concentration levels (10, 25, 50, 100, and 500 µg/mL). In addition, three different concentration levels (30, 150, and 400 µg/mL) covering the entire calibration curve range were prepared to control and validate the method. The standard solutions and control samples were freshly prepared before subjected to the HPLC.

The system suitability test was performed to evaluate the theoretical plate number (NTP), symmetry factor (Sf), and precision of the system by injecting 6 replicates of tHGA solution (100 µg/mL). The system precision was expressed by the relative standard deviation (RSD) for both peak areas and retention times with accepted tolerance of RSD < 2.0%.

The selectivity of the HPLC method was evaluated by injecting a blank solution of the liposomes into the HPLC system and then the chromatogram of the blank solution was compared with the chromatogram of a known concentration of tHGA standard.

The linearity of the HPLC method was assessed on three different days using freshly prepared solution of tHGA, with 10 to 500 µg/mL concentration. The equation y = ax + b was used to express the linear regression, whereby y is the peak area of tHGA; a is the slope of the curve; x is the measured concentration of tHGA, and b is the intercept of the curve on the y-axis. The coefficient of determination (R^2) was used to estimate the linearity of the standard curve.

LLOQ was chosen to be the lowest concentration of the standard curve of which the accuracy and precision were within ± 2%.

Within-run accuracy and precision were determined using three concentration levels with three replicates, each covering the entire range of the analysis. Between-run accuracy and precision were determined using similar samples in three different days. The accuracy was expressed by percent relative error (RE).

Preparation and characterization of liposomes-encapsulated tHGA

The liposomal formulation of tHGA was prepared from proliposomes that contained 50% unsaturated soybean phosphatidylcholines suspended in a specific quantity of food-grade hydrophilic medium (Prolipo™ Duo). The manufacturer's instructions were followed with adjustments made to the dilution steps. Stock solution (128 mg/mL) of tHGA was prepared by dissolving tHGA in DMSO. Then, 250 µL of the stock solution was slowly added into 1 g of proliposomes with stirring. Next, the mixture was hydrated by adding 2 mL of distilled water gradually with stirring for 1 hour. The mixture was finally diluted with water to reach a total volume of 8 mL. The blank liposomal formulation was prepared using the same steps with no addition of tHGA. The following characterization analysis was performed to monitor the physicochemical properties of the formulation.

The entrapment profile of tHGA in the liposomes was determined by a direct measurement of tHGA encapsulated in the formulation. The quantification of tHGA was performed by a validated HPLC method in triplicates. The encapsulation efficiency (EE) was calculated from Equation 2:

$$EE\ \% = \frac{T - F}{T} \times 100 \quad \ldots\ldots\ldots\ldots (2)$$

Where: T is the total amount of tHGA added to the formulation; F is the free non-entrapped tHGA in the liposomes, which is the sum of the amount of undissolved and dissolved tHGA in the formulation.

The amount of undissolved tHGA in liposomes formulation was assessed by centrifuging the prepared samples at 12000 G for ten minutes. The supernatant was then disposed of, and the residual was concentrated with the mobile phase and transferred into HPLC vials for analysis.

The amount of the dissolved tHGA was determined through the complete precipitation of liposomes and free tHGA particles. The prepared liposomal samples were ultra-centrifuged at 300,000 G for 1.5 hours at room temperature using Beckman Optima™ MAX-XP (Beckman, USA). Only the supernatant containing the dissolved tHGA was transferred to HPLC vials for analysis.

The tHGA liposomal sample was analyzed using a dynamic light scattering technique by the Zetasizer Nano S system (Malvern, UK) to measure the size and size distribution of particles in addition to polydispersity index (PDI), and Zeta Potential (ZP) of the prepared liposomes. The procedure following the analysis included dilution of 40 µL of the prepared liposomes in 2 mL distilled water. Then, 10 µL of the sample was transferred into a 12mm o.d. square polystyrene cuvette before subjected to measurement. The analysis was performed at 25°C with a count rate of 96.8 kcps and a measurement position of 2.0 mm.

The stability of prepared liposomes was evaluated according to its EE and particle-size profile. Each of the three prepared liposomal formulations was split into two groups and transferred into airtight polypropylene tubes. The first group was stored at room temperature (~25ºC), and the second group was stored in the

lab refrigerator at (~4ºC). The analysis of the stored samples was performed after one month.

RESULTS

Spectral characteristics and purity of tHGA

Molecular formula $C_{18}H_{24}O_4$; yield 25%; M.P 127-128 °C; UV (λ_{max}/ nm) 290; FT-IR (v_{max}/ cm^{-1}) 3395 (—OH, phenolic), 3302 (C—H, aromatic), 2917 (C—H, alkyl), 1623 (C=O); ^{1}H-NMR (CD_3OD, δ, (ppm)) 1.55 (3H, s, Me), 1.61 (3H, s, Me), 1.73 (3H, s, Me), 1.93 (2H, m), 2.03 (2H, q, J=7.4 Hz), 2.60 (3H, s, CO-Me), 3.18 (2H, d, J=7.1 Hz), 5.05 (1H, t, J=7 Hz), 5.17 (1H, t, J=7.1 Hz), 5.90 (1H, s, ArH); ^{13}C-NMR (CD_3OD, δ, (ppm)) 14.75, 16.25, 20.35, 24.44, 26.35, 31.32, 39.38, 92.98, 103.94, 106.45, 122.75, 124.08, 130.51, 133.27, 160.27, 162.15, 163.41, 203.13; MS $[M-H]^-$ (m/z) 303.1602; MS^2 (m/z) 259.1705 (17), 191.0343 (24), 179.0341 (100), 166.0262 (28), 123.0440 (34), 83.0125 (17), 81.0332 (13).

Table **1**. The precisions represented in RSD for peak area and retention time were 0.41 and 0.40, respectively. NTP was calculated to illustrate the column efficiency quantitatively. The S_f value of the tHGA peak (1.08) indicates that the peak had almost perfect symmetrical Gaussian shape.

The selectivity of the method was evaluated by comparing the blank chromatogram of the liposome formulation with the chromatogram of the tHGA standard at LLOQ. Figure 2 shows that there was no interference between the tHGA peak at 4.1 minute and any other peaks in the blank chromatogram in the HPLC method.

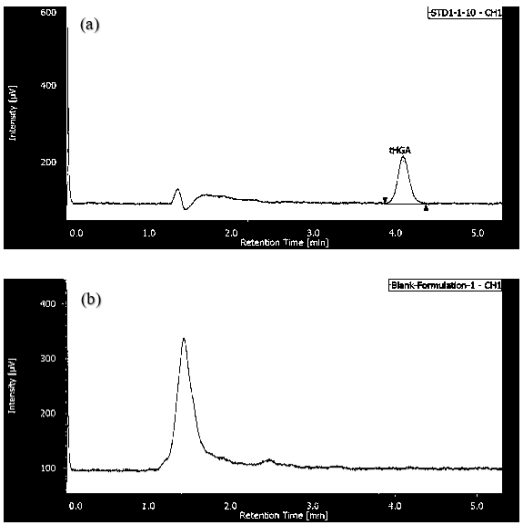


Figure 2: Blank formulation (a) and tHGA standard (b) chromatograms

qNMR test was used to evaluate and verify the purity of tHGA. The resonance selected to quantify tHGA was well resolved from other intramolecular/intermolecular signals and not subjected to deuterium exchange (such as –OH). Dimethyl sulfone ($DMSO_2$) was chosen as an internal calibrant due to its chemical shift value which is distinctive from the chemical shift values of tHGA as well as its stability and availability in high purity. The sharp singlet aromatic proton at δ 5.90 ppm along with $DMSO_2$ (3.01 ppm) signals were chosen to calculate the purity of tHGA. Using Equation 1, the purity of tHGA was calculated to be 98.4%.

HPLC method validation

The system suitability parameters were theoretical plate number (NTP), symmetry factor (S_f), and precision. The mean value of NTP and S_f were 3056 and 1.08, respectively as listed in

The calibration curves exhibited good linearity based on three individual calibration curves constructed on three separate days, over a concentration range of 10 to 500 µg/mL of tHGA. The linear regression equations obtained from calibration curves in day one, two and three were (y = 99.8234x + 214.9649), (y = 100.3468x + 293.0900) and (y = 104.1745x + 381.1917) with coefficient of determinations (R^2) of 1.0000, 0.9999 and 0.9999, respectively. The back-calculation for calibration standards was ranged from -0.63 to 0.5% of the nominal concentration.

The LLOQ was 10 µg/mL, which is the lowest concentration that can be quantified with accuracy and precision of less than 2%, and with symmetrical peak shape (Figure 2 (b)). The mean accuracy and precision of LLOQ samples were -0.63% and 1.00, respectively. This sensitivity is suitable for the determination of the tHGA concentrations in the prepared liposomes.

Table 1: System suitability data for the determination of tHGA (n = 6)

Variable	Peak area	RT (min)	Symmetry factor	Theoretical plates

Mean	11449.2	4.11	1.08	3056
Precision (RSD%)	0.41	0.4		
Range	RSD < 2.0%	RSD < 2.0%	0.8- 1.8	> 2000

Table 2: Within- and between-run accuracy and precision of tHGA

tHGA (µg/mL)	Within-run		Between-run (3 days)		Range (%)
	Accuracy RE %	*Precision RSD %*	*Accuracy RE %*	*Precision RSD %*	
30	1.10	1.20	1.05	1.18	≤ 2
150	-1.69	1.26	-1.97	0.92	≤ 2
400	1.39	0.64	0.70	0.94	≤ 2

Table 3: tHGA entrapment and size profile before and after storing at 4ºC and 25ºC for one month

Test	Size (nm)	PDI	ZP (mV)	EE %
Fresh liposomes	250.76	0.39	-81.1	90.4
Stored for one month at 4 °C	294.14	0.51	-66.8	88.7
Stored for one month at 25 °C	325.92	0.78	-59.6	61.2

Within-run accuracy and precision were estimated in three replicates at three QC levels (30, 150, and 400 µg/mL). As shown in Table **2**, the within-run accuracy was less than 1.69% and precision was less than 1.26%, which are within the tolerable value of ± 2%. Between-run precision and accuracy were estimated in three replicates at three concentrations of tHGA (30, 150, and 400 µg/mL). The between-run accuracy and precision ranged from -1.97 to 1.05% and 0.92 to 1.18 %, respectively.

In vitro physicochemical characteristics of the tHGA-loaded liposomes

The HPLC method was successfully applied to quantify the dissolved and undissolved tHGA in the prepared liposomes. The encapsulation efficiency of tHGA-loaded liposomes was 90.4%. The size profile of the prepared liposomes was evaluated by the measurement of particle size (diameter) and size distribution expressed by the polydispersity index (PDI) besides the zeta potential value. The mean diameter of the liposome particles, PDI, and ZP were 250.8 nm ± 7.9, 0.39 ± 0.01, and -66.8 mV ± 2.61, respectively.

The stability of the prepared encapsulated tHGA under two different storage conditions was evaluated with respect to their compound and size profile. The results listed in Table 3 demonstrate that there were no significant changes in the compound entrapment and size profile for the samples stored at refrigerated temperatures (~4 ºC) for one month. In contrast, the samples stored at room temperature showed significant changes in the compound entrapment and size profile. The results indicate that the liposome-encapsulated tHGA has good stability when stored at ~4 ºC for at least one month while having poor stability when stored at room temperature.

DISCUSSION

Although reference standards can be purchased from official sources, without official source (like in tHGA case), a reference standard has to be of the highest purity compound which can be achieved through reasonable effort, and careful characterization [3]. Hence in this study tHGA was fully characterized to confirm its identity, quality, and purity.

Analytical method validation is an essential requirement to confirm that the data generated from the method is reliable and reproducible. In this study, the HPLC was validated according to ICH guidelines. The basic parameters evaluated were system suitability, linearity and rang, LLOQ, precision, and accuracy. The system suitability results demonstrated the suitability of the HPLC system used in this study to produce reliable data for the quantification of tHGA. the calibration curve results demonstrated excellent relationships between the tHGA concentration and the response of the HPLC which proved the ability of this method to accurately measure an unknown concentration of tHGA in samples.

Accuracy and precision are the most critical parameters in analytical method validation, thus the results of the present work demonstrate that this analytical method is accurate and precise to quantify tHGA.

Zareen *et al* (2013) reported an HPLC method for the quantification of tHGA simultaneously with the other two benzene derivatives in *Melicope ptelefolia* leaves. The retention time of tHGA in that method was 17.35 minutes [20]. In the present method, the mobile phase used was simple and effective to elute tHGA in short retention time (4.1 minutes) and total run time (5.5 min) with excellent peak shape. The simplicity and speed of the method along with high accuracy and precision, made this method suitable for the routine analysis of tHGA in the liposomal formulation and other related studies.

The practical use of liposomes demands monitoring of the physicochemical properties of the liposomes such as its size, polydispersity, encapsulation efficiency, and stability [21]. The high encapsulation efficiency achieved indicates that this formulation is effective for dissolving and entrapping a high amount of tHGA. In addition, the high EE value implies that only a small amount of phospholipid is required to entrap tHGA, which decreases the cost of liposomes production and reduces the side effects associated with high dose of phospholipids. Different factors could have contributed to the high EE value obtained from the tHGA-loaded liposomes, such as the composition of proliposomes used, the approach adopted in the preparation of the liposomes, the hydration time, and the physicochemical properties of tHGA. PDI value of the prepared tHGA-loaded liposomes was 0.4 indicating narrow distribution of particle size of the liposome particles. ZP values over 60 mV (positive or negative) imply excellent stability because the highly charged particles increase the electric repulsion and avoid aggregation of particles [22,23]. In this study, tHGA-loaded liposomes shows a high ZP value of -66.8 mV, indicating excellent stability for the formulation.

CONCLUSION

tHGA has been successfully synthesized and then purified by a two-step purification procedure. The compound, with high purity (98.4 %), can be used as an analytical reference standard. Characterization of tHGA has also been achieved and its identity confirmed. In addition, a simple and effective method to prepare and characterize liposomal tHGA formulation has been accomplished. The developed HPLC method shows good linearity, high precision and accuracy. The stability of the encapsulated-tHGA liposomes was adequately good for at least one month when stored at ~4 ºC, but poor when stored at room temperature. Based on these findings, the developed formulation is effective for dissolving and entrapping a high amount of tHGA which helps to overcome its poor solubility and low bioavailability.

DECLARATIONS

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Conflict of interest

The authors declare that there is no conflict of interest with regard to this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Khozirah Shaari: Conceptualization, Supervision, Funding acquisition, Writing- Reviewing and Editing, Yamen Alkhateeb: Investigation, Data curation, Writing- Original draft preparation. Qais B. Jarrar: Resources, Faridah Abas: Supervision, Yaya Rukayadi: Supervision.

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